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RESEARCH ARTICLE

Biodiversity of Ectoparasites and Molecular Detection of *Bartonella* in Ectoparasites Infesting *Rhinolophus Affinis* in Yunnan Province, China

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ABSTRACT

Bats, with their robust immune system, frequently serve as hosts for viruses and bacteria. *Bartonella* spp. are transmitted by blood-sucking arthropods and are widely distributed among various mammalian species globally. The present study focused on the molecular detection of *Bartonella* spp. From ectoparasites infesting widely distributed *Rhinolophus affinis* in Southwest China. Briefly, the bats were captured from four districts, namely Xundian, Jinning, Lufeng, and Mouding and ectoparasites collected from these bats. The ectoparasites were identified based on morphological characteristics, and the biodiversity of ectoparasites infesting bats in the study area was elucidated through spatial distribution and analysis of dominant species. Further, DNA was extracted from ectoparasites and three targeted genes (*ftsZ*, *gltA*, and *rpoB*) of *Bartonella* were amplified using conventional PCR. Sixty *R. affinis* bats were captured with an impressive ectoparasites infestation rate of 90.00%. A total of 10 species were identified comprising 1,412 ectoparasites, with the dominant species being *Eyndhovenia euryalis* (Y = 0.099, m^*/m = 2.439), *Macronyssys tieni* (Y = 0.563, m^*/m = 2.040), *Macronyssys dechangensis* (Y = 0.024, m^*/m = 1.815), *Ixodes vespertilionis* (Y = 0.026, m^*/m = 2.146), and *Stylidia fraterna* (Y = 0.052, m^*/m = 1.436) (one species of bat fly), all of which exhibited aggregated distribution on the host's body surface. Notably, the detection of 172 ectoparasites in the bats revealed that all positive cases of *Bartonella* were found in *S. fraterna*. This high prevalence may be attributed to the unique reproductive strategy of bat flies, which facilitates vertical transmission of *Bartonella*. Therefore, bat flies may act as potential vectors for *Bartonella* transmission. This study deepens understanding of the ectoparasite diversity of *R. affinis* and broadens our knowledge of the geographical distribution of this pathogen.

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INTRODUCTION

The order Chiroptera, which encompasses bats, constitutes the second-largest mammalian group, with 21 families, 230 genera, and over 1,400 species worldwide, representing approximately 20% of all mammals (Teeling *et al*., 2005). As the most widely distributed mammals, bats frequently interact with both humans and livestock owing to their exceptional flight capabilities and diverse diets. This close proximity has led to the transmission of various zoonotic pathogens, particularly viruses, to humans, including SARS-CoV, Hendra, Nipah, Ebola, and Rabies, posing significant threats to public health and safety (Rodhain, 2015; Moratelli and Calisher, 2015). Studies have indicated that, with the exception of rabies, bat viruses generally require intermediate hosts or vectors to transmit these pathogens to humans (Plowright *et al*., 2015). Bats are also known to carry a diverse array of ectoparasites, including mites, ticks, bat flies, fleas, and lice, which can serve as vectors for various pathogens. These parasites, while feeding on bats, facilitate the transmission of these pathogens to other hosts, including humans, thereby playing a significant role in the epidemiological dynamics between species (Dietrich *et*

al., 2016; Lee *et al*., 2021). Investigating the infection status of these parasites and the pathogens they carry is crucial for understanding the origins of zoonotic diseases and the mechanisms behind the cross-species transmission facilitated by bats.

Bartonella, a group of Gram-negative rod-shaped bacteria belonging to the Bartonellaceae family within the Rhizobiales order, primarily parasitize red blood cells and endothelial cells in mammalian hosts (Urushadze *et al*., 2017). They are predominantly transmitted through hematophagous arthropods. This genus is known to cause a range of zoonotic diseases, including Cat-scratch disease (CSD) caused by *Bartonella henselae*, Trench fever linked to *Bartonella quintana*, and Carrion's disease caused by *Bartonella bacilliformis*. Furthermore, *Bartonella elizabethae* has been implicated in causing endocarditis (Kim *et al*., 2009). Recent research has uncovered numerous novel *Bartonella* species in bat populations and their ectoparasites (Han *et al*., 2022). The observed association between bat-borne *Bartonella* and human endocarditis has gained attention, with the detection of serological evidence in high-risk communities. This reinforces the strong likelihood of zoonotic transmission from these bat-associated species and their parasites, highlighting the significant epidemiological implications of these findings (Veikkolainen *et al*., 2014; Bai *et al*., 2018).

Rhinolophus affinis (Horsfield, 1823), a prevalent insectivorous bat species found in East, South, and Southeast Asia, including China, is particularly abundant and widespread in these regions (Ith *et al*., 2015). Since the emergence of the novel coronavirus, studies of its origin have suggested that *R. affinis* in Southeast Asia may act as a potential natural host. Yunnan Province, strategically located as a gateway between China and several Southeast Asian nations, supports a substantial population of this species. The combination of the strong flight capabilities and migratory tendencies of *R. affinis* raises concerns about the potential for cross-regional and inter-species transmission of associated pathogens (Li *et al*., 2021).

This study aimed to determine the biodiversity of ectoparasites infesting bats in Yunnan Province. their potential to harbor pathogens, such as *Bartonella*, with the ultimate goal of informing targeted disease control strategies for bat-borne pathogens in the region. The findings may contribute valuable insights into our understanding of zoonotic disease transmission dynamics.

MATERIALS AND METHODS

Bat capturing, collection, and identification of ectoparasites: At the cave entrance, a mist net was set up to capture bats leaving for foraging and returning to the cave. Species identification of *R. affinis* was done based on the diagnostic features outlined in the Handbook of Mammals of China (Shaoying, 2022) and the distinctive acoustic characteristics of their echolocation calls. Sterile forceps were used to collect ectoparasites from the surface of the bats, which were then placed in corresponding cryotubes. After the experiment, the bats were released back to their original location. A subset of the parasites was chosen for species identification, which involved the preparation of slides using slide glass, cover glass, and Hoyer's solution. These slides were subsequently subjected to blast drying in a Bluepard DHG-9240A oven (China) for one month. Species identification was conducted using a Leica DM3000LED biological microscope (Germany), with reference to established literature and classification resources (Deng, 1993; Oskar, 1967; Poon *et al*., 2023). While the remaining ectoparasites were used for pathogen detection analysis. This study was ethically approved by the Dali University Ethics Committee (Approval No: MECDU-202104-27).

Biodiversity of ectoparasite infestating bats

Analysis of ectoparasitic infestation: Each *R. affinis* individual was examined to quantify the number of unique parasite species and their respective individuals. To assess the parasite load, we employed the following statistical measures: Constituent ratio (*Cr*), Prevalence (*P*), Mean abundance (*MA*), and Mean intensity (*MI*).

$$C_r = \frac{N_i}{N} \times 100\% \qquad P = \frac{H_m}{H} \times 100\%$$

$$MA = \frac{N_i}{H} \qquad MI = \frac{N_i}{H_m}$$

These parameters were calculated using the following formulas: In the equation, N_i = the number of individuals of a certain type of ectoparasite; N = the total number of ectoparasites; H_m = the number of bats infected with a certain type of ectoparasite; and H = the total number of captured bats.

Analysis of dominant species: The species dominance index *Y* was used to assess the dominant species in the ectoparasite community on the surface of *R. affinis*.

$$Y = \frac{n_i}{N} \times f_i$$

The calculation formula is as follows: In the above formulas, n_i represents the number of individuals of a certain parasite collected at all sampling points; N is the total number of parasite individuals collected at all sampling points; f_i is the frequency of occurrence of a certain parasite at all sampling points. When $Y > 0.02$, the species is considered dominant in the community.

Spatial distribution analysis: The Patchiness index (m^*/m) and Index of *K* value (*K*) was used to measure the spatial distribution pattern of the dominant gamasid mite species among the different individuals of the host.

$$\frac{m^*}{m} = \frac{m + [\frac{\sigma^2}{m} - 1]}{m} \qquad K = \frac{m}{[\frac{\sigma^2}{m} - 1]}$$

The calculation formulas are as follows: In the above formulas, m represents the mean number of a certain parasite infecting each individual bat, σ^2 is the variance of the number of a certain parasite infecting each individual bat, and m^* is the average crowding.

Table 1: Primers and the PCR reaction conditions used in this study.

Targetspecies	Gene	Primers	Sequences (5′-3′)	PCR products (bp)	PCR conditions	Reference
Bartonella	*gltA*	gltA-F	GCTATGTCTGCATTCTATCA	751	95°C/5 min; 40 cycles: 95°C/30 s,	(Han *et al*., 2022)
		gltA-R	GATCYTCAATCATTTCTTTCCA		48°C/30 s, 72°C/30 s; 72°C/5 min.	
	rpoB	rpoB-F	CGCATTGGCTTACTTCGTATG	852	95°C/5 min; 40 cycles: 95°C/30 s,	
		rpoB-R	GTAGACTGATTAGAACGCTG		48°C/30 s, 72°C/30 s; 72°C/5 min.	
	ftsZ	ftsZ-F1	ATTAATCTGCAYCGGCCAGATAT	791	95°C/5 min; 40 cycles: 95°C/30 s,	
		ftsZ-R1	TCATCAATRGCVCCAAARAT		48°C/30 s, 72°C/30 s; 72°C/5 min.	

(1) When m*/m<1, it is determined as a uniform distribution; m*/m = 1, it is a random distribution; m*/m>1, it is determined as an aggregated distribution.
(2) When K<0, it is determined as a uniform distribution; when K→∞, it is determined as a random distribution; K>0, it is determined as an aggregated distribution.

Pathogen detection in ectoparasites

DNA extraction and conventional PCR amplification: Prior to DNA extraction, bat flies were immersed in distilled water for 5-10 minutes to remove any remaining ethanol from their surfaces. Subsequently, ectoparasites were placed in collection tubes adhering to the principle of one parasite per tube, and then were minced using sterile blades. Following the guidelines provided by the manufacturer, the TIANamp Genomic DNA Kit from TIANGEN in Beijing, China, was utilized for the extraction procedure. Samples were stored at -20°C for later use. Subsequently, the extracted DNA samples were used as templates for PCR amplification to confirm the presence of *Bartonella*. The PCR reaction mixture was 20μl, comprising 6μl of deionized water, 10μl of master mix, 1μl each of forward and reverse primers, and 2μl of template DNA. The primer sequences and reaction conditions are shown in Table 1.

Sequence analysis: The PCR-amplified positive products were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. The obtained data were viewed using Geneious Prime to check the peak profiles. Only high-quality sequences were selected to construct phylogenetic trees. If the nucleotide similarity between sequences was 100%, one sequence was chosen for analysis. Subsequently, the target sequences were compared with existing sequences using the Nucleotide Basic Local Alignment Search Tool (BLASTn) on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The best tree model was determined using MODELS in MEGA X. Then, multiple sequence alignments were performed on the respective reference sequences in MEGA X, and phylogenetic trees based on each gene were constructed using the Neighbour Joining (NJ) method, with a Bootstrap value of 1000 replicates for each gene. Finally, figtree and AI were used to edit and visualize each phylogenetic tree. All obtained sequences have been uploaded to the NCBI database.

RESULTS

Identification and biodiversity of ectoparasites: A survey of 23 bat sampling points in central Yunnan captured 60 adult *R. affinis* from four counties and districts (Xundian, Jinning, Lufeng, and Mouding), with 15 males and 45 females. Among the 60 *R. affinis* captured, 54 were found to be infected with ectoparasites on their bodies, resulting in a high infection rate of 90.00%. A total of 1412 ectoparasites were collected and identified as follows: *E. euryalis* of the family Spinturnicidae, *Paraperiglischrus strandimanni* of the genus Paraperiglischrus, *Spinturnix kolenatii* of the genus Spinturnix, *M. tieni*, *M. dechangensis*, *Macronyssys. zhijinensis*, and *Macronyssys. coreanus* of the family Macronyssidae, *I. vespertilionis* of the genus Ixodes, *S. fraterna* of the family Nycteribiidae, and *Brachytarsina* sp. of the family Streblidae, totaling 10 species. The composition and distribution of these families, genera, and species are presented in Table 2.

Table 2: Infestation of the ectoparasites on *R. affinis*.

Ectoparasite	Number	*Cr*/%	*P*/%	*MA*	*MI*
Spinturnicidae	284	20.11	76.67	4.73	6.17
Eyndhovenia	279	19.76	73.33	4.65	6.34
E. euryalis	279	19.76	73.33	4.65	6.34
Paraperiglischrus	3	0.21	3.33	0.05	1.50
P. strandimanni	3	0.21	3.33	0.05	1.50
Spinturnix	2	0.14	3.33	0.03	1.00
S. kolenatii	2	0.14	3.33	0.03	1.00
Macronyssidae	935	66.21	80.00	15.58	19.48
Macronyssys	935	66.21	80.00	15.58	19.48
M. tieni	795	56.30	80.00	13.25	16.56
M. dechangensis	136	9.63	63.33	2.27	3.58
M. zhijinensis	3	0.21	1.67	0.05	3.00
M. coreanus	1	0.07	1.67	0.02	1.00
Ixodidae	74	5.24	46.67	1.23	2.64
Ixodes	74	5.24	46.67	1.23	2.64
I. vespertilionis	74	5.24	46.67	1.23	2.64
Nycteribiidae	98	6.94	61.67	1.63	2.65
S. fraterna	98	6.94	61.67	1.63	2.65
Streblidae	21	1.49	16.67	0.35	2.10
Brachytarsina sp.	21	1.49	16.67	0.35	2.10
Total	14.12	100	90.00	23.53	28.24

Among the 10 ectoparasites found on the bodies of *R. affinis*, *E. euryalis* (*Y*=0.099, *P*=73.33%, *Cr*=19.76%), *M. tieni* (*Y*=0.563, *P*=80.00%, *Cr*=56.30%), *M. dechangensis* (*Y*=0.024, *P*=63.33%, *Cr*=9.63%), *I. vespertilionis* (*Y*=0.026, *P*=46.67%, *Cr*=5.24%), and *S. fraterna* (*Y*=0.052, *P*=61.67%, *Cr*=6.94%) collectively accounted for a high proportion of 97.87% in the ectoparasite community of *R. affinis*. These five species are considered dominant species on the bodies of *R. affinis* (see Fig. 1, Tables 2 and 3). As shown in Table 3, the calculated values of *m*/m* and *K* were all higher than the border values, 1 or 0 for the spatial distribution pattern of all the five dominant gamasid mite species, *E. euryalis*, *M. tieni*, *M. dechangensis*, *I. vespertilionis* and *S. fraterna*. Therefore, it has been observed that these five dominant parasitic species exhibit aggregated distributions across different individuals of the host (Table 3).

***Bartonella* prevalence**: In 2023, 60 *R. affinis* individuals were captured from Moding County, Chuxiong Yi Autonomous Prefecture, and Xundian Hui and Yi

Table 3: Spatial distribution patterns of dominant ectoparasite species on *R. affinis*.

Dominant Parasite Species	Species dominance index *Y*	*K* parameter of the negative binomial distribution	Cluster Index *m**/m*	Spatial Pattern Recognition
E. euryalis	0.099	0.695	2.439	Aggregated Distribution
M. tieni	0.563	0.962	2.040	Aggregated Distribution
M. dechangensis	0.024	1.227	1.815	Aggregated Distribution
I. vespertilionis	0.026	0.872	2.146	Aggregated Distribution
S. fraterna	0.052	2.296	1.436	Aggregated Distribution

Fig. 1: *R. affinis* is infected by the *I. vespertilionis.*

Table 4: Sampling information and prevalence of *Bartonella* in tick and bat flies.

Sample type	Species	Number of samples	Number of positive samples
Bat fly	*S. fraterna*	98	*ftsZ*: 18.60%(32/172); *gltA*: 15.70%(27/172); *rpoB*:11.63% (20/172)
Tick	*I. vespertilionis*	74	0.00

Autonomous County, Kunming City. In total 172 ectoparasites (*S. fraterna* and *I. vespertilionis*) were collected. In this study, three genes were screened for the *Bartonella* in each sample. The molecular detection of *Bartonella* in 172 specimens revealed a positivity rate of 18.60% (32/172) for *ftsZ*, 15.70%(27/172) for *gltA*, and 11.63% (20/172) for *rpoB*. Further details are provided in Table 4.

Molecular features of *Bartonella*: The results demonstrated that *S. fraterna* carry multiple strains of *Bartonella*, with most clustering with *Bartonella* previously reported in bats and bat flies. Based on partial *ftsZ* gene sequences, the *Bartonella* detected in this study could be classified into following 5 types: sequence type 2 clustered with *Bartonella* from bat ectoparasites, *I. vespertilionis*, in Romania; sequence types 1, 3, 4, and 5 clustered with *Bartonella* from bat flies and bats from China, Thailand, and Ghana, respectively (Fig. 2).

In addition, La Scola *et al*. proposed a *Bartonella* species identification standard based on the *gltA* and *rpoB* gene sequences in 2003: If the similarity percentages of the *gltA* segment (327 bp) and *rpoB* segment (825 bp) to sequences of validated species were less than 96.0% and 95.4%, respectively, the *Bartonella* isolates should be considered as new species (La Scola *et al*., 2003). According to this criterion, the *Bartonella* isolates detected in this study based on the *gltA* partial gene sequences could be classified into 5 genotypes. Genotype 1 clustered with *Bartonella* from bats in Kenya; genotype 2 clustered with *Bartonella* from bat flies in South Korea; genotype 3 clustered with *Bartonella* from *Urva auropunctata* in St. Kitts and Nevis and *Bartonella washoensis*; genotype 4 clustered with *Bartonella* from bats in Vietnam, and genotype 5 clustered with *B. hansaea*, showing high node support. Based on *rpoB* partial gene sequences, the 4 genotypes detected in this study clustered with *Bartonella* isolated from bats in Kenya and Georgia. Among these, genotypes defined by *gltA* gene III and *rpoB* gene IV showed nucleotide similarity below the cut-off values (*gltA* 96% and *rpoB* 95.4%) compared to existing *Bartonella* in the gene bank, indicating existence of new *Bartonella* species (Fig. 3 and 4).

Based on a 751bp segment of the *gltA* gene, this study found that the *Bartonella* genotypes 3 and 5 were clustered with *B. washoensis* and *B. henselae*, respectively. *B. washoensis* is associated with human endocarditis, while *B. henselae* can cause cat-scratch disease. However, homology analysis revealed only 87-90% nucleotide identity between them, which is below the cutoff value of 96%. Therefore, it can only be said that they are more closely related in terms of phylogeny to *B. washoensis* and *B. henselae*.

DISCUSSION

Over the past few decades, the recurrent emergence and re-emergence of global infectious diseases have significantly jeopardized human health and survival. Among these, zoonotic diseases transmitted by wildlife are particularly prevalent and devastating (Gay *et al*., 2014). Bats and their ectoparasites serve as natural reservoirs for a wide array of viral and bacterial pathogens. Following the outbreaks of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and the novel coronavirus (COVID-19) in 2019, numerous studies have implicated bats as the source of these coronaviruses (Zhou *et al*., 2020; Ruiz-Aravena *et al*., 2022). Owing to their carriage of numerous zoonotic agents, bats have garnered substantial interest within the fields of preventive medicine and pathobiology. Pathogens harbored by bats can transmit to humans through direct contact, intermediate hosts, or vectors, thereby giving rise to diverse zoonotic infections (Plowright *et al*., 2015). Consequently, elucidating potential intermediate hosts or vectors for bat-borne pathogens and conducting in-depth studies on their pathogen load are pivotal components of epidemiological investigations. This knowledge is instrumental in guiding the monitoring of local disease vectors, as well as the prevention and control of associated diseases.

This study captured *R. affinis* in four distinct locations in central Yunnan, including Xundian, Jinning, Lufeng, and Mouding. These sites, being geographically dispersed, provide insights into the ectoparasite infestation

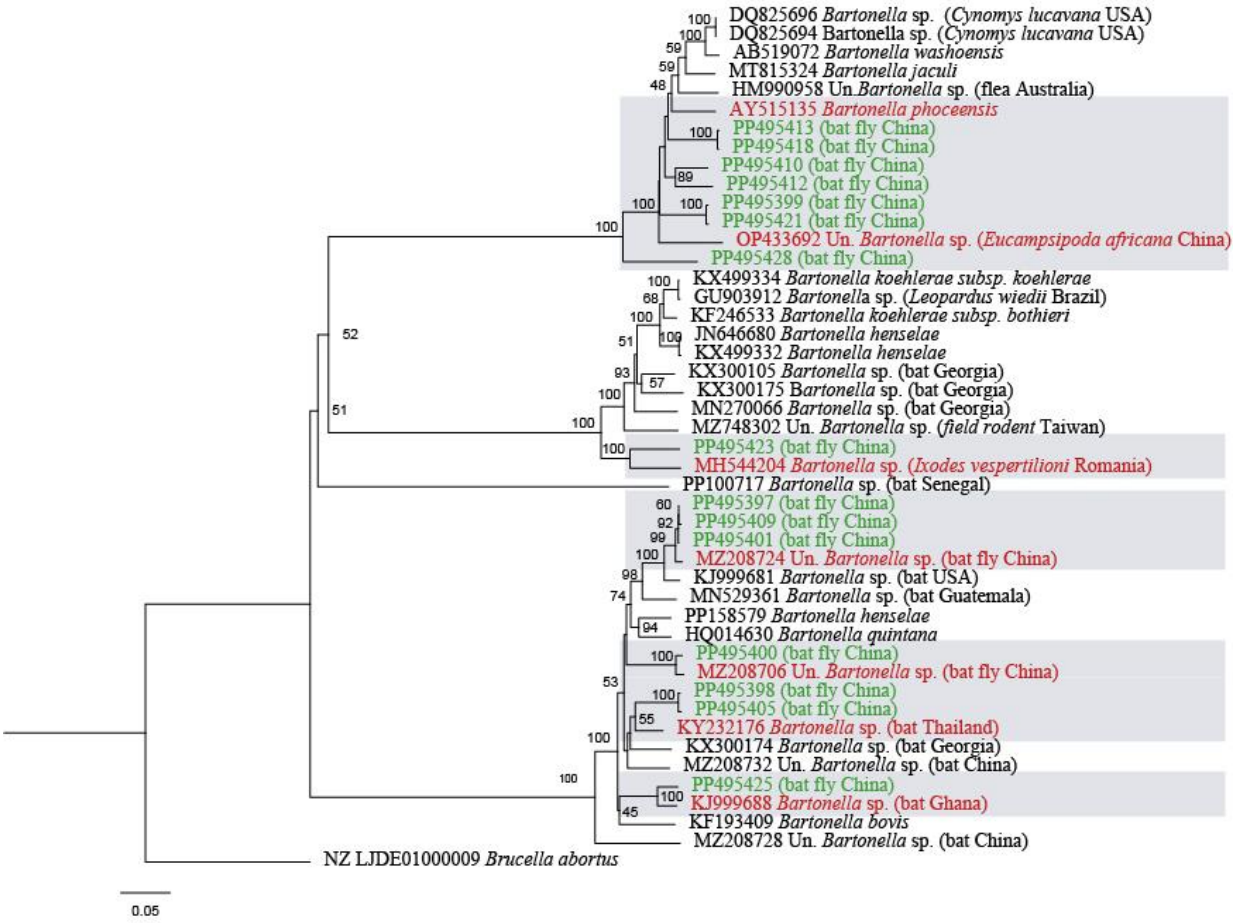

Fig. 2: Phylogenetic analysis of bat ectoparasites *Bartonella* based on the *ftsZ* gene (791 bp). Phylogenetic tree was constructed using the Neighbour-Joining method in MEGA X.

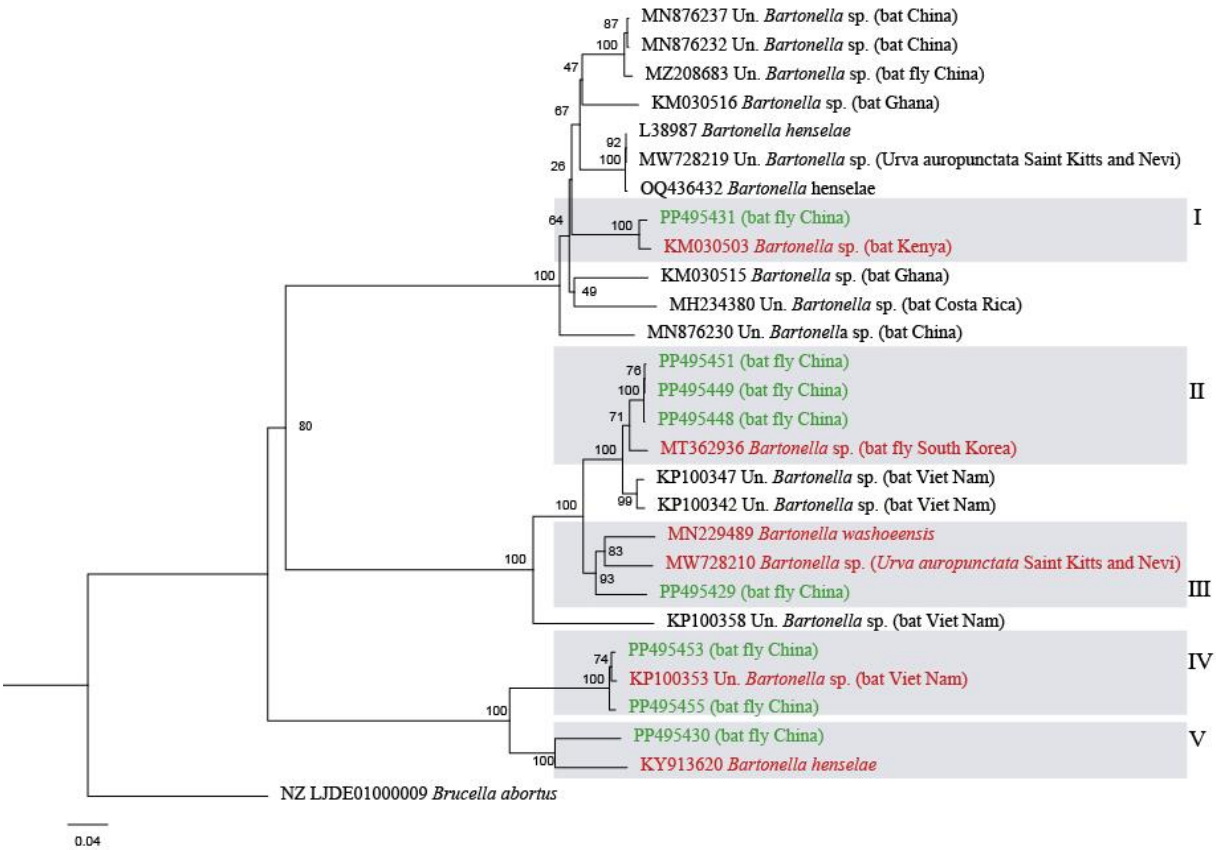

Fig. 3: A phylogenetic tree constructed with the *Bartonella gltA* gene sequences using the Neighbour-Joining method.

patterns of *R. affinis* in the region. Our findings indicate a widespread infestation of ectoparasites on *R. affinis*, with an infection rate of up to 90.00% and an average of 23 parasites per bat. A total of three major categories, encompassing 10 species, were identified, including gamasid mites, ticks, and bat flies. The ectoparasite diversity on *R. affinis* was higher than that observed in *Rhinolophus sinicus*, *Ia io*, *Myotis chinensis*, and *Miniopterus fuliginosus* (Fan *et al*., 2022; Zhang *et al*., 2023; Zhang *et al*., 2023; Yang *et al*., 2023), but significantly lower compared to other small mammals like *Rattus brunneusculus* (Lv *et al*., 2021). This disparity is postulated to be linked to the high host specificity of bat ectoparasites. Future studies will broaden the sampling scope to deepen our understanding of the ectoparasite diversity on *R. affinis*.

The collected ectoparasites, including *E. euryalis*, *M. tieni*, *M. dechangensis*, *I. vespertilionis*, and *S. fraterna*, exhibited high prevalence and abundance, suggesting that they are the primary species on the body of *R. affinis*. The assessment of spatial distribution is essential in arthropod ecology. Spatial distribution patterns are typically classified into three categories: uniform, random, and aggregated. Indices such as the Patchiness index and the Index of *K* value are often employed to evaluate the spatial distribution patterns of animal populations (Liu *et al*., 2020). In this study, five dominant ectoparasitic species were identified as exhibiting aggregated distributions among various individuals of *R. affinis*. The aggregated distribution model suggests that the infestation levels of these five dominant parasites are unevenly distributed across different hosts. Some hosts may harbor a high number of parasites, whereas others may have very few or none. This aggregated distribution is a common pattern observed in many ectoparasites and may confer advantages for parasite survival, mating, and reproduction (Huang *et al*., 2013; Yin *et al*., 2021). This clustering also increases the likelihood of pathogen transmission within or between host species. Notably, *E. euryalis* and *I. vespertilionis*, two dominant ectoparasites of *R. affinis*, have previously been found on *R. sinicus* in the same region (Fan *et al*., 2022). Two potential explanations for this co-occurrence are: first, the close cohabitation of *R. affinis* and *R. sinicus* in caves may facilitate the transfer of ectoparasites between them; second, both species belong to the Rhinolophus genus within the Vespertilionidae family, and our research group has observed shared ectoparasites among multiple Rhinolophus species, though further investigation is needed to understand the underlying mechanisms. In Poland, reports indicated that *I. vespertilionis*, an occasional host for humans, has been observed biting cave explorers (Piksa *et al*., 2013), potentially transmitting pathogens such as *Bartonella*, *Rickettsia*, and tick-borne encephalitis (TBE) through ticks, thereby posing a health risk to humans (Piksa *et al*., 2016; Kuang *et al*., 2022; Krčmar *et al*., 2022). *M. dechangensis*, first identified by Zhang Fuguo in 1981 in Dechang County, Sichuan Province, China, was initially found on *R. affinis* and is now known to inhabit Yunnan Province, broadening our understanding of its geographical

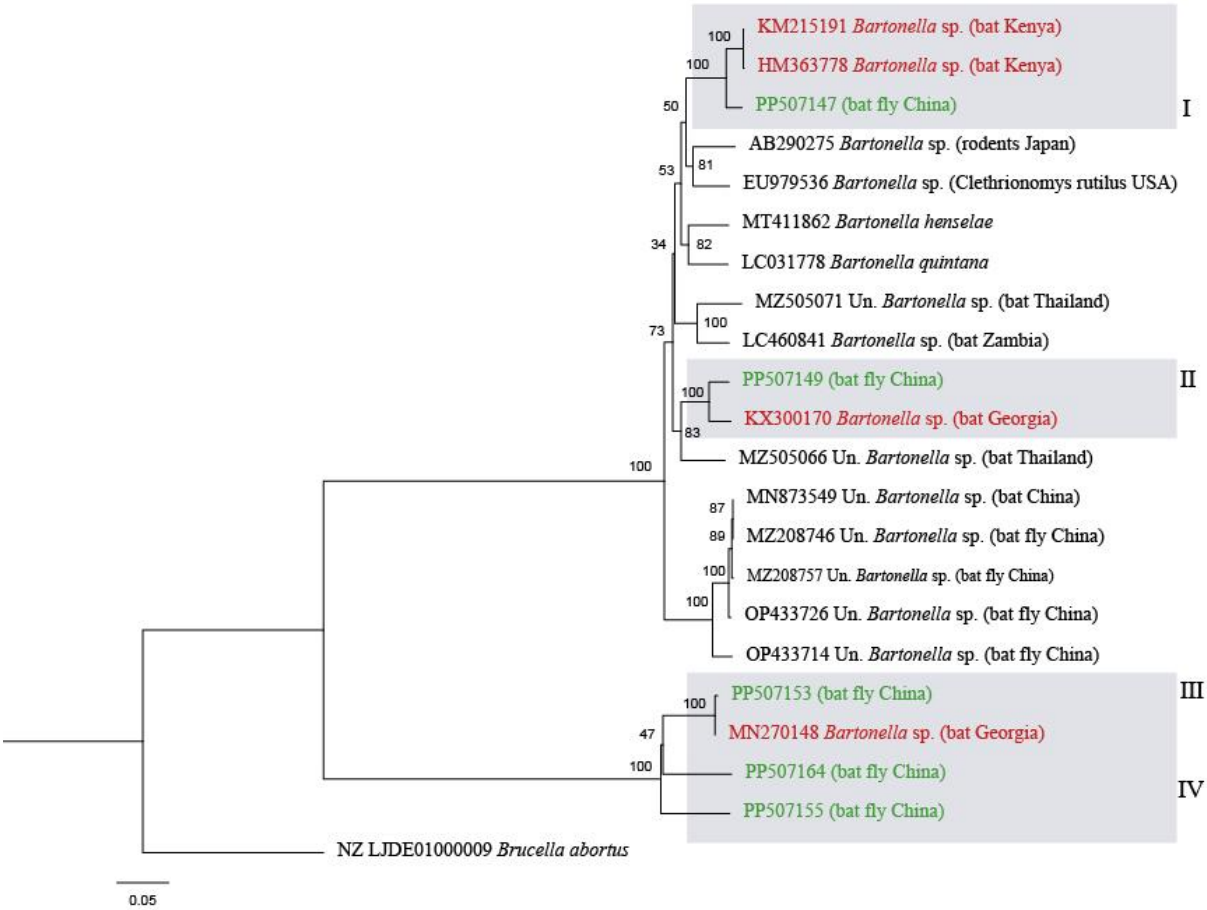

Fig. 4: A phylogenetic tree constructed with the *Bartonella rpoB* gene sequences using the Neighbour-Joining method.

distribution. *M. tianae*, previously recorded on bats in Fujian, Taiwan, and Vietnam, still lacks clarity regarding its specific host. The first documentation of *S. fraterna* on *Rhinolophus ferrumequinum* in Jinan City, Shandong Province, China, and Japan, has been expanded upon by our study, confirming that both *M. tianae* and *S. fraterna* primarily parasitize *R. affinis*, thus elucidating their natural hosts and expanding their known geographic range.

In recent years, the surge of novel coronavirus has intensified scholarly interest in bat parasites and their pathogenic potential. However, current pathogen detection methods often lack specificity, focusing on broad bat parasite groups in limited regions without differentiating between species. Systematic investigations into the pathogenicity of these carried pathogens are lacking, hindering the development of targeted control strategies for potential spillover events. To address this gap, our study aimed to enhance understanding of bat ectoparasite-borne pathogens by examining *Bartonella* carriage in *R. affinis* in Yunnan Province, China. *Bartonella*, a Gram-negative bacterium that thrives in mammalian reservoirs and facilitates transmission through blood-feeding arthropods (Veikkolainen *et al*., 2014), has been increasingly detected directly from these vectors using molecular biology techniques. Our investigation revealed *Bartonella* in *S. fraterna* and *I. vespertilionis* collected from Yunnan Province, China, with positive gene detection rates of *gltA* (18.6%), *ftsZ* (15.7%), and *rpoB* (11.63%). Intriguingly, all positive samples corresponded to *S. fraterna*, while *I. vespertilionis* exhibited a zero-positive rate. The high infection rate in *S. fraterna* could be linked to its unique reproductive strategy (adenotrophic vivipary), where larvae develop from fertilized eggs within the female and feed on glandular secretions, facilitating vertical transmission of *Bartonella* (Yang *et al*., 2023). Other studies have also reported similar pathogens in ticks that parasitize bats (Leulmi *et al*., 2016; Hornok *et al*., 2019). The absence of *Bartonella* in *I. vespertilionis* in our study may be attributed to host species differences or geographical variations. In 2019, *B. washoensis* (GenBank accession number: MN229489) was isolated from the mitral valve tissue of a 75-year-old German female patient with endocarditis. Sequencing and MLST analysis revealed that this isolate shared 99% identity with a *B. washoensis* strain isolated from red squirrels from China. Furthermore, the PP495429 isolate, obtained from the body surface of a *S. fraterna*, clustered phylogenetically with the German female isolate MN229489 in this study. Another isolate, PP495430, clustered with *B. henselae* (KY913620) isolated from cat fleas. However, a perplexing finding was that nucleotide homology between these isolates, as determined by NCBI BLAST, was only 90-87%, which is significantly lower than the species identification threshold for *Bartonella* (96% for the *gltA* gene), indicating a contradiction. This discrepancy might be explained by the common occurrence of recombination events in the *gltA* gene among bacterial populations (Paziewska *et al*., 2011).

Our study uncovers a high diversity of ectoparasites on *R. affinis* in Yunnan Province, with *S. fraterna* harboring multiple *Bartonella* genotypes, in line with previous observations. The resemblance between *Bartonella* strains detected in bat ectoparasites and those found in bats implies that hematophagous behavior may facilitate species-to-species transmission (Yang *et al*., 2024), emphasizing the potential role of ectoparasites as vectors. To fully comprehend their role in pathogen transmission, further research is needed. Future studies should employ metagenomic sequencing (mNGS) and bioinformatics to uncover a broader spectrum of pathogens carried by bats and their ectoparasites. To validate the pathogenic potential of newly discovered pathogens, isolation, cultivation, and pathogenicity experiments are crucial for assessing their effects on other animals and investigating the role of parasites in facilitating cross-species transmission by bats.

Conclusions: This study investigated the ectoparasite infestation on *R. affinis* in Yunnan Province, China, and further examined *Bartonella* carriage in ectoparasites. The findings contribute to enriching the scarce data on bat ectoparasites in China, providing crucial references for subsequent research. Additionally, the study offers theoretical guidance for the prevention and control of vector-borne diseases in the region. This research provides valuable insights for the management of vector-borne diseases.

Conflict of interest: We have no conflict of interest related to this work.

Authors contributions: Xiaoyan Zheng & Xianzheng Zhang designed the study and wrote the manuscript, Xiaobin Huang & Xinke Yue edited the manuscript and provide financial help. Yujuan Wang analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Data availability statement: The data presented in the study have been deposited in the NCBI database repository under the following accession numbers: *ftsZ* for PP495397-PP495428; *gltA* for PP495429-PP495455; *rpoB* for PP507147-PP507166.

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Ethical statement: The bat capture and ectoparasite collection methods involved in this experiment were approved by the Medical Ethics Committee of Dali University (approval no: MECDU-202104-27), and were conducted following the Chinese Association for Laboratory Animal Sciences and the Institutional Animal Care and Use Committee (IACUC) protocols.

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